THE BACTERICIDAL AND HEMOLYTIC POWERS OF "PARAFFIN" PLASMA AND OF SERUM.*

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In 1901 Gengou[1] published the details of experiments which showed that plasma had very little or no bacteriolytic action *in vitro* on a variety of organisms which were readily destroyed by serum. This result was advanced as an experimental proof of the correctness of Metchnikoff's theory that complement does not exist in the plasma of the circulating blood but is contained in the white blood corpuscles, and that complement is only found in serum because changes take place in the leukocytes during coagulation which lead to its liberation. Gengou laid stress on the fact that his results were obtained with plasma to which no anticoagulant had been added and which so far at least was in a condition comparable to that in which it exists in the body. This was regarded as a point of importance by some workers, and eight papers have since appeared in which Gengou's experiments were repeated with plasma to which no addition was made. Of these, one, that of Herman, confirms Gengou's results without qualication; the others just as clearly and decisively show that plasma has as much bacteriological and hemolytic power as serum.

On comparing the technic adopted by Gengou with the methods employed by those who have repeated his work, there is found to be one important difference. Gengou prepared mammalian plasma by drawing it directly from a vessel through a paraffined canula into paraffin-lined vessels and centrifuging at 0° C. until it was entirely free from cells. When this plasma was removed from the cooled centrifuge tubes and allowed to attain the ordinary room temperature it quickly clotted. He compared the fluid expressed from this clot with serum derived from the natural coagulation of the whole blood. So in reality he did not directly compare plasma with serum, but the serum from a cell-free plasma with the serum from blood.

Those who deny or confirm the reliability of his results have for the most part adopted another method of procedure. They have used the cell-free plasma itself and not the serum from it. Now this introduces an entirely new factor, the factor

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[1] *Ann. de l'Inst. Pasteur*, 1901, 15, p. 232.

of coagulation. For it may be taken as certain that it is impossible to carry out any bacteriolytic or hemolytic experiment with mammalian paraffin plasma without intercurrent coagulation. This is a point the importance of which appears to a great extent to have escaped the attention it deserves. Thus Dömeny[1] in his paper does not mention coagulation at all. Sweet[2] states that the plasma coagulated during the course of the experiment. Hewlett,[3] who worked with goose plasma, which is much more stable than mammalian plasma, does not say whether it coagulated or not. Löwit and Schwartz,[4] indeed, recognized the possible influence of this factor and lay no stress on their bacteriolytic work with bird's plasma, since they found that coagulation always occurred. Lambotte,[5] who also used bird's plasma, says that it coagulated during the bacteriolytic reaction. Herman,[6] who worked with mammalian plasma, says nothing on this point. Falloise[7] and Schneider[8] alone have followed Gengou's method and used the serum expressed from the coagulation of cell-free plasma. Their experiments are the only ones which accurately reproduce Gengou's, and their results are diametrically opposed to his. Nevertheless the faith of the adherents to Metchnikoff's theory is not seriously shaken. They object that Falloise and Schneider during the process of withdrawing the blood and centrifuging it had damaged the leukocytes so that they gave up their content of complement to the plasma. Falloise and Schneider have, indeed, by careful and painstaking work given evidence that the leukocytes were not injured to any appreciable extent, but without avail. Gengou's results are still relied on and it is supposed that by more careful technic he was enabled to obtain a plasma free not only from cells but also from more than traces of leukocytic disintegration. This is a position which has not been made absolutely untenable.

If in a repetition of these experiments the results of Falloise and Schneider were confirmed, the position would have remained for all practical purposes unchanged. The use of plasma itself instead of the serum of plasma is open to the same objections and is further complicated by the possible influence of the process of coagulation on bacteriolysis and hemolysis. Both of these methods were therefore rejected and at Dr. Ritchie's suggestion an attempt, ultimately successful, was made to obtain an unaltered plasma so stable that it would not coagulate during the course of the experiments. Such experiments should, I think, be regarded as conclusive, for the fact that a plasma will remain uncoagulated after being kept for an hour and a half or more at a temperature of 37° C. is in itself conclusive proof that there has been no appreciable amount of damage to the white blood corpuscles. The integrity

[1] *Wien. klin. Wchnschr.*, 1902, 40, p. 105.
[2] *Centralbl. f. Bakt.*, I, Orig., 1903, 33, p. 208.
[3] *Arch. f. exper. Path. u. Pharmak.*, 1903, 49, p. 307.
[4] *Ztschr. f. Heilkunde*, 1903, 24, pp. 205, 301.
[5] *Centralbl. f. Bakt.*, I, Orig., 1903, 34, p. 453.
[6] *Bull. de l'Acad. Roy. de Méd.*, 1904, p. 157.
[7] *Bull. de l'Acad. Roy. de Méd.*, 1905, p. 230.
[8] *Arch. f. Hyg.*, 1908, 65, p. 305.

of the leukocytes up to the time when they are removed from the plasma is the essential condition of the continued fluidity of that plasma. When they have previously been injured, even to a very slight extent, the plasma promptly clots. These experiments also make it possible to estimate the effect of coagulation occurring during the bacteriolytic reaction. This is of some importance, for it will allow one to determine the force of the objection to experiments in which this complication occurred.

METHODS.

The preparation of plasma.—The circulating blood remains fluid because of the absence of one of the essential factors in coagulation. This substance, usually termed thrombokinase, is liberated when cells are injured or destroyed. If blood were obtained directly from a vessel without coming in contact with the injured cells of the wound surface and the formed elements in it could be removed without injuring them, a plasma would be obtained which would not spontaneously coagulate. In spite of numerous attempts and of elaborate precautions against damaging the blood cells, no one has as yet succeeded in preparing such a plasma from mammalian blood. On the other hand, with care it is possible with bird's blood. Presumably their blood cells are more resistant to trauma than mammalian blood cells. Such bird's plasma will remain indefinitely fluid if kept at room temperature in a paraffined vessel. In my experience, however, when it is kept in contact with a foreign body at a temperature of 37° C. it will in time coagulate. This indicates that the plasma is not absolutely free from thrombokinase, although the amount must be extremely small, since it is only able to act under the most favorable conditions as to temperature and with the adjuvant action afforded by the presence of a foreign body. Even under these circumstances the period during which the plasma remains uncoagulated is amply sufficient to allow of bacteriolytic or hemolytic experiments being carried out. The real difficulty arises from the fact that this period of fluidity is very much shortened by the addition of bacteria and to a less extent of red blood corpuscles. Their action in this respect is probably a double one. There is the liberation as they become

lysed of any thrombokinase they contain and there is the accelerating action they may exert on the action of the thrombokinase in virtue of their effect as foreign bodies. If they had contained more than traces of active thrombokinase the experiment would have been impossible. But the red blood corpuscles in contradistinction to the leukocytes contain no thrombokinase. If the plasma and leukocytes of the blood from which the corpuscular suspension was prepared were as far as possible removed by washing, it ought to contain only extremely small amount of kinase. A-priori considerations would lead one to expect that kinase would be found in extracts of bacteria, but the existence of a certain degree of specificity in the action of kinase allowed one to hope that it might not induce a rapid coagulation. These considerations made it plain from the commencement that it would be a matter of difficulty to keep the plasma from coagulating during the experiments and as a matter of fact this was found to be the case, and it was only after repeated modifications that a procedure was arrived at by means of which the plasma remained absolutely free from clot for the required time.

The plasma was obtained from cock's blood. The femoral artery was exposed, separated from the tissues of the wound by rubber tissue, and thoroughly washed with warm normal saline. After placing a clip on the proximal end and ligaturing below, the artery was opened and the interior washed. A paraffined glass canula was then introduced and as the clip was removed the canula was pushed into the vessel beyond the part formerly compressed by the clip. After the first few cubic centimeters had run out, the blood was collected in paraffin tubes and as soon as possible centrifuged. This was carried out with a powerful electrical centrifuge. After 10 minutes there was macroscopically a complete separation of the corpuscles from the plasma. The upper part of this plasma was carefully pipetted off, placed in other paraffined tubes and centrifuged again for 20 minutes. Again only the upper part of this plasma was removed and it was this part which was used. Microscopically it was entirely cell free.

In each case part of the blood was collected separately to serve as a source of serum with which to compare the plasma. Some fine

sand was shaken up with it in order to injure the leukocytes and to induce a more rapid coagulation than would otherwise have occurred. There is almost no retraction of the clot in bird's blood and very little serum separates even after centrifuging, but sufficient was obtained by compressing the clot by means of a lead weight.

In the case of plasma or serum used in bacteriolytic experiments all these procedures were carried out under aseptic conditions.

It was found that in the bacteriolytic experiments it was necessary to use tubes lined with sterile block paraffin and closed with paraffined corks, but this precaution could be dispensed with in the hemolytic experiments if care were taken to make the corpuscular emulsion as free as possible from kinase. Rabbit's citrated blood was repeatedly washed by centrifuging in saline solution and at each repetition the upper half of the precipitated corpuscles was removed. In this way an emulsion of red blood corpuscles was obtained which was completely free from white blood corpuscles and platelets. Traces of thrombokinase probably remained adhering to the red cells in spite of washing, but not in sufficient amount to cause coagulation before the hemolytic reaction was ended.

THE COMPARATIVE BACTERIOLYTIC POWER OF PLASMA AND SERUM.

Three series of paraffined tubes were prepared containing respectively plasma, serum, and broth. Progressive dilutions of an emulsion of *B. coli* were added to each and after incubating at 37° C., 0.5 c.c. of each tube was mixed with melted agar and plated. The colonies were counted after 24 hours at 37° C.

Plasma, serum, and broth = 1 c.c.
Emulsion = 2 loops in 10 c.c. broth.
Amount of emulsion or diluted emulsion added = 0.1 c.c.
Time of incubation at 37° C. = 2 hours.

Dilution of Emulsion	No. of Colonies with Plasma	No. of Colonies with Serum	No. of Colonies with Broth
Emulsion	Uncountable	Uncountable	Uncountable
1–10	Uncountable	Uncountable	Uncountable
1–100	1,800	1,740	Uncountable
1–1,000	200	281	2,340
1–10,000	41	55	568
1–100,000	11	8	108

Plasma, serum, and broth = 1 c.c.
Emulsion = 1 loop in 10 c.c. broth.
Amount of diluted emulsion added = 0.1 c.c.
Time of incubation at 37° C. = 1½ hours.

Dilution of Emulsion	No. of Colonies with Plasma	No. of Colonies with Serum	No. of Colonies with Broth
1–10	1,680	2,128	Uncountable
1–100	436	264	2,576
1–1,000	105	102	380
1–10,000	13	25	113
1–100,000	0	4	9
1–1,000,000	0	1	7

These results show that bird's plasma has as much bacteriolytic power on *B. coli* as has the serum.

THE COMPARATIVE HEMOLYTIC POWER OF PLASMA AND SERUM.

Advantage was taken of the fact that cock's serum is hemolytic to rabbit's corpuscles.

Time of incubation at 37° C. = 1½ hours.

Corpuscles, 1 per cent suspension	Plasma	Saline	Result	Corpuscles, 1 per cent suspension	Serum	Saline	Result
1 c.c.	0.07 c.c.	0.93 c.c.	C	1 c.c.	0.07 c.c.	0.93 c.c.	C
1 c.c.	0.06 c.c.	0.94 c.c.	C	1 c.c.	0.06 c.c.	0.96 c.c.	+++
1 c.c.	0.05 c.c.	0.95 c.c.	++	1 c.c.	0.05 c.c.	0.95 c.c.	++
1 c.c.	0.04 c.c.	0.96 c.c.	+	1 c.c.	0.04 c.c.	0.96 c.c.	+
1 c.c.	0.03 c.c.	0.97 c.c.	0	1 c.c.	0.03 c.c.	0.97 c.c.	

Time of incubation at 37° C. = 1 hour.

Corpuscles, 1 per cent suspension	Plasma	Saline	Result	Corpuscles, 1 per cent suspension	Serum	Saline	Result
1 c.c.	0.15 c.c.	0.85 c.c.	C	1 c.c.	0.15 c.c.	0.85 c.c.	C
1 c.c.	0.1 c.c.	0.9 c.c.	++++	1 c.c.	0.1 c.c.	0.9 c.c.	C
1 c.c.	0.09 c.c.	0.91 c.c.	++++	1 c.c.	0.09 c.c.	0.91 c.c.	C
1 c.c.	0.08 c.c.	0.92 c.c.	+++	1 c.c.	0.08 c.c.	0.92 c.c.	++++
1 c.c.	0.07 c.c.	0.93 c.c.	++	1 c.c.	0.07 c.c.	0.93 c.c.	+++
1 c.c.	0.06 c.c.	0.94 c.c.	+	1 c.c.	0.06 c.c.	0.94 c.c.	++
1 c.c.	0.05 c.c.	0.95 c.c.	+	1 c.c.	0.05 c.c.	0.95 c.c.	+
1 c.c.	0.04 c.c.	0.96 c.c.	0	1 c.c.	0.04 c.c.	0.96 c.c.	+
1 c.c.	0.025c.c.	0.975c.c.	0	1 c.c.	0.025c.c.	0.975c.c.	0

As regards hemolytic power, therefore, no noticeable distinction was apparent between plasma and serum. The slight differences which were found lie well within the limits of error of the method.

THE EFFECT OF INTERCURRENT COAGULATION ON BACTERIOLYSIS AND HEMOLYSIS WITH PLASMA.

In the above experiments there was no trace of coagulation in the plasma at the end of the time of incubation. In some experiments, however, coagulation did occur and the question arose as

to whether this process had in any way modified the bacteriolysis or hemolysis. When the plasma had coagulated it was of course impossible to plate it, and there was therefore no means of measuring accurately the degree of bacteriolysis which had occurred. But a rough idea was obtained by making cultures on agar from the clotted plasma, serum, and broth, in order to see with what dilution of emulsion the plasma or serum had become sterile. This method did not give constant results, but no indication was obtained that the clothing had either helped or hindered the bacteriolysis.

It was however easy to determine the influence of coagulation on hemolysis. Three series of tubes containing plasma and a suspension of corpuscles were prepared. In the first, coagulation was induced in the plasma by the addition of thrombokinase (a dilute watery testicular extract) before the corpuscles were added. They were of course unable to mix with the clot and formed a separate layer above it. In the second series coagulation was induced during the reaction by the addition of thrombokinase. In the third series instead of thrombokinase the same amount of water was added and the plasma remained throughout uncoagulated. Controls showed that the thrombokinase had no hemolytic action. It was necessary to use as a diluting fluid for the corpuscles and for the plasma a saline solution containing 0.06 per cent of calcium chloride, as otherwise the tubes containing the smaller amounts of plasma did not coagulate with kinase, because the calcium concentration had in them been reduced below the minimum amount which is necessary.

Rabbit corpuscles = 1 per cent suspension in a solution containing 0.9 per cent NaCl and 0.06 per cent CaCl.
Saline solution = 0.9 per cent NaCl and 0.06 per cent CaCl.
Thrombokinase = 0.1 c.c.
Time of incubation = 1½ hours.

Plasma Coagulated before Addition of Corpuscles				Plasma Coagulated during Hemolysis				Plasma Uncoagulated			
Corp.	Plasma	Sodium	Result	Corp.	Plasma	Saline	Result	Corp.	Plasma	Saline	Resu
c.c....	0.1 c.c.	0.9 c.c.	Hemolysis	1 c.c..	0.1 c.c.	0.9 c.c.	C	1 c.c..	0.1 c.c.	0.9 c.c.	C
c.c....	0.09 c.c.	0.91 c.c.	"	1 c.c..	0.09 c.c.	0.91 c.c.	+++	1 c.c..	0.09 c.c.	0.09 c.c.	+++
c.c....	0.08 c.c.	0.92 c.c.	"	1 c.c..	0.08 c.c.	0.92 c.c.	++	1 c.c..	0.08 c.c.	0.92 c.c.	++
c.c....	0.07 c.c.	0.93 c.c.	"	1 c.c..	0.07 c.c.	0.93 c.c.	+	1 c.c..	0.07 c.c.	0.93 c.c.	+
c.c....	0.06 c.c.	0.92 c.c.	0	1 c.c..	0.06 c.c.	0.94 c.c.	0	1 c.c..	0.06 c.c.	0.94 c.c.	0

The result was thus the same in each case. Whether the plasma was coagulated before or during the hemolysis or remained uncoagulated, 0.07 c.c. produced some hemolysis and 0.06 c.c. caused none. In the first case, where the clot and corpuscles remained separate, the degree of hemolysis was difficult to estimate, but there was no doubt about the last tube showing lysis, for the least trace of hemoglobin could be seen diffusing into the clear saline solution left by the settling of the corpuscles onto the clot.

Coagulation has therefore no effect on hemolysis.

THE EFFECT OF THE CLOT ON THE COMPLEMENT-CONTENT OF THE SERUM.

A possible objection to the above comparative experiments with plasma and serum is the fact that before testing they were not subjected to exactly the same conditions. The serum was in contact with the clot, which might conceivably increase or diminish the amount of complement. Indeed, Ainly Walker[1] and Henderson Smith[2] have described slight changes in the amount of bacteriolytic complement of serum separated at different intervals of time from the clot. In general they maintain that there is a gradual increase in complement for the first five or six hours, which they ascribe to a slow liberation of complement from leukocytes entangled in the fibrin. The later diminution which occurred is referred to absorption of complement by the clot. The serum in the above experiments was removed from contact with the clot after an interval varying from three to six hours, so that according to Ainly Walker and Henderson Smith it should have contained about its maximum amount of complement. But bird's blood has not been investigated as regards this point, and it seemed advisable to do so, since it was possible that a rapid absorption of complement by the clot might be the cause of the serum containing no more complement than plasma.

[1] *Jour. Hyg.*, 1903, 3, p. 52.

[2] *Proc. of the Roy. Soc.*, 1906, Series B, 79, p. 378.

RABBIT'S CORP. 1 PER CENT	BIRD'S SERUM	TIME DURING WHICH THE SERUM HAD BEEN IN CONTACT WITH THE CLOT					
		½ Hour	1½ Hours	2 Hours	3 Hours	4 Hours	6 Hours
1 c.c.	0.09 c.c.	C	C	C	C	C	C
1 c.c.	0.08 c.c.	C	C	++++	C	++++	++++
1 c.c.	0.07 c.c.	++++	++++	++++	++++	++++	++++
1 c.c.	0.06 c.c.	++++	++++	++++	++++	++++	++++
1 c.c.	0.05 c.c.	+++	+++	+++	+++	+++	+++
1 c.c.	0.04 c.c.	++	++	++	++	++	++
1 c.c.	0.03 c.c.	+	+	+	+	+	+
1 c.c.	0.02 c.c.	o	o	o	o	o	o

There was thus no noteworthy difference in the hemolytic power of serum removed at these various intervals of time from the clot, and one may conclude that in bird's blood the clot has no effect on the amount of complement, within the first six hours at least. In view of the apparent difference between bird's blood and mammalian blood the same experiment was repeated with rabbit's serum.

A rabbit was bled, and after mixing the blood was poured into eight centrifuge tubes and allowed to clot. At various intervals thereafter each tube was centrifuged to separate the serum, and the amount of complement in it was determined.

Corpuscles = 1 per cent suspension of ox-blood corpuscles.
I.B. = serum of rabbit immunized against ox-blood corpuscles.

CORP.	I.B.	COMPLEMENT	TIME DURING WHICH THE COMPLEMENT HAD BEEN IN CONTACT WITH THE CLOT						
			½ Hour	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	8 Hours
1 c.c.	0.005 c.c.	0.2 c.c.	C	C	C	C	C	C	C
1 c.c.	0.005 c.c.	0.1 c.c.	++++	++++	++++	++++	++++	++++	C
1 c.c.	0.005 c.c.	0.05 c.c.	++++	++++	+++	+++	+++	+++	++++
1 c.c.	0.005 c.c.	0.04 c.c.	+++	+++	++	++	++	++	+++
1 c.c.	0.005 c.c.	0.03 c.c.	++	++	+	+	+	++	++
1 c.c.	0.005 c.c.	0.02 c.c.	+	+	o	+	+	+	+
1 c.c.	0.005 c.c.	0.01 c.c.	o	o	o	o	+	+	+
1 c.c.	0.005 c.c.	0.005 c.c.	o	o	o	o	o	o	o

Here also there was no appreciable variation, and I have thus been unable to confirm the statements as to variations in the amount of complement in serum, either with bird's or with rabbit's serum.

CONCLUSIONS.

Unaltered bird's plasma has as much bacteriolytic and hemolytic power as serum. This equality cannot be accounted for by supposing that the clot has absorbed a large part of the complement in the serum, since it has been shown that no such absorption occurred.

Nor, on the other hand, can these results be put aside as inconclusive because of the possibly complicating effect of coagulation, since no coagulation took place. They show that the hypothesis that complement is derived from the leukocytes when they are injured is not correct. For if the white blood corpuscles had been damaged even slightly, thrombokinase would have been liberated and the plasma would have coagulated. No more delicate test of the degree of leukocytic injury than the length of time during which a plasma will remain fluid can be devised. Gengou maintains that the amount of complement in plasma is proportional to the degree of leukocytic disintegration. Yet in bird's plasma, in which, as has been shown, there was less cell injury than in his plasma, there was nevertheless as much complement as in serum, whereas on his hypothesis there should have been scarcely a trace.

Once the alleged greater accuracy of Gengou's technic is disproved, the matter becomes one to be decided from the evidence of results. This evidence is very much in favor of the view that complement exists in the circulating plasma and that it is not derived from injured leukocytes. Gengou's results have indeed been confirmed by one observer, but as he worked with very small amounts of plasma and serum, with hanging drop preparations of plasma and serum for example, there are possible sources of error which might explain his findings. The seven others who have investigated this point are unanimous in coming to the conclusion that plasma contains as much complement as serum. Their results are none the less to be accepted as valid evidence canceling that brought forward by Gengou, although coagulation may have taken place in the plasma during the experiments, since I have shown that the process of coagulation has no influence on the course of bacteriolytic or hemolytic reactions. And when, as in the experiments described in this paper, it is shown that plasma derived from blood in which the amount of cell injury has been minimal contains as much complement as serum from blood in which every influence favored the destruction of the leukocytes, I think it is no longer possible to hold that complement is derived from injured leukocytes. This, then, is another point to be added to all the other evidence which goes to show that complement is present in the circulating plasma.